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Noninvasive Methods for Determining Lesion Depth From Vesicant Exposure

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Before sulfur mustard (HD) injuries can be effectively treated, assessment of lesion depth must occur. Accurate depth assessment is important because it dictates how aggressive treatment needs to be to minimize or prevent cosmetic and functional deficits. Depth of injury typically is assessed by physical examination. Diagnosing very superficial and very deep lesions is relatively easy for the experienced burn surgeon. Lesions of intermediate depth, however, are often problematic in determining the need for grafting. This study was a preliminary evaluation of two noninvasive bioengineering methodologies, laser Doppler perfusion imaging (LDPI) and indocyanine green fluorescence imaging (ICGFI), to determine their ability to accurately diagnose depth of sulfur mustard lesions in a weanling swine model. Histological evaluation was used to assess the accuracy of the imaging techniques in determining burn depth. Six female weanling swine (8–12 kg) were exposed to 400 μ l of neat sulfur mustard on six ventral sites for 2, 8, 30, or 60 minutes. This exposure regimen produced lesions of varying depths from superficial to deep dermal. Evaluations of lesion depth using the bioengineering techniques were conducted at 24, 48, and 72 hours after exposure. After euthanasia at 72 hours after exposure, skin biopsies were taken from each site and processed for routine hematoxylin and eosin histological evaluation to determine the true depth of the lesion. Results demonstrated that LDPI and ICGFI were useful tools to characterize skin perfusion and provided a good estimate of HD lesion depth. Traditional LDPI and the novel prototype ICGFI instrumentation used in this study produced images of blood flow through skin lesions, which provided a useful assessment of burn depth. LDPI and ICGFI accurately predicted the need for aggressive treatment (30- and 60-minute HD lesions) and nonaggressive treatment (2- and 8-minute HD lesions) for the lesions generated in this study. Histological evaluation confirmed the accuracy of the assessment. The ICGFI instrument offers several advantages over LDPI including real-time blood flow imaging, low cost, small size, portability, and not requiring the patient to be repositioned. A negative, however, is the need for intravenous dye injection. Although this would not be an issue in a hospital, it may be problematic in a mass casualty field setting. Additional experiments are required to determine the exposure time necessary to produce a graded series of partial-thickness HD lesions and to optimize instrumental parameters. The data generated in this follow-on study will allow for a full assessment of the potential LDPI and ICGFI hold for predicting the need for aggressive treatment after HD exposure. The lasting message is that objective imaging techniques can augment the visual judgment of burn depth. (*J Burn Care Res* 2007;28:275–285)

Accurate wound depth assessment is important in dictating the degree of aggressive treatment to minimize or prevent cosmetic and functional deficits

from a vesicant lesion. In chemical burns, depth of injury typically is assessed by physical examination. Surface appearance, the pinprick test to assess pain,

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of the authors and are not necessarily endorsed by the US Army. This*

*research complied with the Animal Welfare Act and implementing
Animal Welfare Regulations and adhered to the principles noted in
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the “blanch-capillary return test” to evaluate microcirculation, and surface temperature difference between burned and unburned skin are often used in diagnosis of depth.¹ When using these methods, diagnosing very superficial and very deep burns is relatively easy for the experienced burn surgeon. Burns of intermediate depth often are problematic in determining the need for aggressive treatment including grafting.

Noninvasively examining cutaneous blood flow, using available bioinstrumentation, can greatly assist the physician in making depth-of-injury determinations. Laser Doppler perfusion imaging (LDPI) and indocyanine green fluorescence imaging (ICGFI) may prove to be very valuable tools in diagnosing depth of vesicant injuries. This study was a preliminary evaluation of these two noninvasive bioengineering methodologies to determine their ability to accurately diagnose the depth of sulfur mustard (HD) lesions in a weanling swine model.

Laser Doppler flowmetry and LDPI have been used for prolonged, noninvasive monitoring of tissue viability and wound healing and for the assessment of peripheral vascular disease, inflammation, ischemia, reperfusion, skin graft acceptance (take), and burn depth.^{2–17} LDPI has proven useful in delineating the areas of damage that need to be débrided and avoiding areas with sufficient blood flow. Brown et al¹⁸ found that LDPIs of vesicant vapor burns on the backs of swine correlated well with histopathological findings (thrombosis and necrosis of subepidermal capillaries) between 1 hour and 7 days after exposure and suggested that clinical management decisions on how to treat early vesicant burns could be aided by LDPI. Chilcott et al¹⁹ used several noninvasive bioengineering methods to monitor wound healing in a large white pig model for 7 days after exposure to HD and Lewisite vapors. They concluded that, although reflectance colorimetry and transepidermal water loss measurements could provide quantitative, noninvasive methods for determining the efficacy of candidate treatment regimens, neither is comparable with the prognostic capabilities of LDPI. Graham et al²⁰ found LDPI to be useful in examining blood flow in grafted and ungrafted sites after the treatment of deep dermal/full-thickness liquid HD injuries in a weanling swine model.

LDPI is currently rather time consuming if there are multiple sites to be evaluated and/or large images to be collected at high resolution. The length of scanning procedures could be decreased by increasing scanning speed (thus decreasing flux resolution), decreasing the size of the scan area, and/or decreasing the number of lines scanned within the scanning area

(scan resolution). Improvements in the technology that will speed up LDPI without compromising image resolution are being developed.

Indocyanine green fluorescence imaging (ICGFI) also has shown promise in determining burn depth based upon microcutaneous blood flow. The fluorescence of intravenous ICG has been shown to estimate burn depth in small animals.²¹ In contrast to fluorescein fluorescence,²² ICG fluorescence is capable of distinguishing superficial and deep partial-thickness burns from full-thickness burns. The fluorescence intensity of ICG decreases exponentially with burn depth for burns of similar age.²³ ICG fluorescence was used to estimate burn depth in a porcine model, using healing as an endpoint.²⁴ A prototype imaging system with a diagnostic algorithm, developed at the Wellman Laboratories of Photomedicine, Boston, MA, was used to accurately diagnose burns that healed within 21 days with minimal scarring from those that took longer to heal by secondary means. Measurements were made on burns created 2, 24, 48, and 72 hours before imaging. The algorithm was shown to be dependent on the age of the burn and independent of the location of the burn. This technology showed promise in plastic surgical applications,^{25,26} and accurate determination of thermal burn depth in humans.^{25,27,28}

ICG administration is a minimally invasive procedure that requires the placement of an intravenous line. ICG has been approved by the Food and Drug Administration for use in humans to determine cardiac output, hepatic function and liver blood flow, and for ophthalmic angiography. The dose of ICG (IC-GREEN, Akorn, Inc., Buffalo Grove, IL) is typically 0.2 to 0.8 mg/kg administered intravenously as a bolus through a central or peripheral venous line. The smallest dose that gives optimal fluorescence signals should be used. Multiple boli may be required for examination of large TBSA. The total dose of dye injected should be kept below 2 mg/kg, as recommended by the dye's manufacturer. Reported LD50s after intravenous administration in animal studies are 60 to 80 mg/kg in mice, 50 to 70 mg/kg in rats, and 50 to 80 mg/kg in rabbits.

According to the manufacturer, after intravenous injection, the dye rapidly binds to plasma proteins (principally albumin). It undergoes no significant extrahepatic or enterohepatic circulation with negligible renal, peripheral, pulmonary, or cerebrospinal uptake. The dye is removed from the plasma almost exclusively by hepatic parenchymal cells and secreted into the bile for excretion. Because the dye solution contains sodium iodide (not more than 5.0%), its use is contraindicated in patients with a history of allergy to iodides. Anaphylactic or urticarial reactions have

been reported in patients with or without history of allergy to iodides.

The advantages that this technology has over LDPI include greatly increased speed of image capture and ability to examine microcutaneous blood flow in real time. Multiple images over large areas can be captured in a relatively short period of time. Images are typically collected 5 to 10 minutes after ICG injection to allow uptake and distribution. The dye is then excited (780 nm), and the resultant fluorescence emission (825 nm) immediately captured and saved by a computer and analyzed for burn to normal skin fluorescence ratio. ICG binds strongly to plasma globulins, limiting both extravasations within burn-injured vascular epithelia and extravascular transport to areas nearby.²³ Large signals are thought to be the result of vasodilation and hyperemia, and smaller signals are thought to be attributable to vascular occlusion and edema.^{21,23}

MATERIALS AND METHODS

Animal Model

Six female Yorkshire Cross pigs (weanlings), *Sus scrofa*, weighing 8 to 12 kg, were used (Country View Family Farms, Harrisburg, PA). They were quarantined upon arrival for 7 days and screened for evidence of disease before use. In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources, National Research Council, in accordance with the stipulations mandated for an AAALAC-accredited facility. Animals were supplied tap water ad libitum and fed approximately 300 g of Teklad Mini-Swine Breeder Sterilizable Diet (Harlan Teklad 7037, Harlan Teklad, Madison, WI) twice a day. Animals were housed individually in 4 × 6-ft pens with slatted aluminum floors. The animal holding room was maintained at 21 ± 2°C with 50 ± 10% relative humidity using at least 10 complete air changes per hour of 100% conditioned fresh air. Animal rooms were maintained on a 12-hour light/dark full-spectrum lighting cycle with no twilight.

Sulfur Mustard Exposure

Eighteen to 24 hours before agent exposure, each animal was sedated by intramuscular injections of xylazine HCl (Rompun, Bayer Healthcare, Leverkusen, Germany; 1.0 ml @ 20 mg/ml) and a combination of tiletamine HCl and zolazepam HCl (Telazol, Fort Dodge Laboratories, Fort Dodge, IA; weighed out in equal parts, reconstituted to 100 mg/ml, and 0.5 ml

administered), and hair was removed from the ventral surface by chemical depilation using MAGIC Fragrant Shaving Powder (Carson Products Co., Savannah, GA).

On the morning of exposure, the pigs were anesthetized to a surgical plane of anesthesia with Rompun (2.2 mg/kg) and Telazol (6 mg/kg) intramuscularly. Six exposure sites were set up on the ventral surface, three sites per side parallel to and approximately 2.5 cm lateral to the teat line and located between the axillary and inguinal areas. A plastic template was used for even spacing and consistent anatomical positioning of the sites among animals. In addition to the exposure sites, two nonexposure control sites were located along the ventral midline, one located near the axillary area and the other near the inguinal area. Tape assemblies (5 cm × 5 cm) were prepared out of double-sided carpet tape and duct tape, with a 2.9-cm diameter hole punched through the center of each tape assembly. A circle of Whatman No. 2 glass microfiber filter paper (3.8 cm in diameter) was sandwiched between the carpet tape and the duct tape and then centered over the 2.9-cm hole. A small bead of cyanoacrylate adhesive was placed along the periphery of the hole on the duct tape, and a rubber O-ring (31-mm inner diameter) glued onto the template. The templates were placed approximately 6 cm apart, center to center, onto the ventral surface, one centered in each of the six exposure sites. The pig was placed in an agent hood in dorsal recumbency, supported by a stainless-steel pig sling. A therapeutic heating pad (Gaymar Industries, Inc., Orchard Park, NY), with the circulating water temperature set at 41°C, was placed under the animal during the exposure period to minimize hypothermia. Using a pipette (Gilson, Pipetman P-1000), 400 µl of undiluted HD was placed on each filter paper. A solid polytetrafluoroethylene cap liner (PTFE; 0.38-mm thick, sized for a 28-mm cap) was placed over the filter paper, followed by an appropriately sized rubber stopper (#7) to occlude the site and ensure complete contact of the wetted filter paper with the skin. The purpose of the O-ring was to keep the PTFE disk and rubber stopper in place.

An animal was exposed for 2, 8, 30, or 60 minutes. The 2- and 8-min sites were exposed one at a time using a 300-g weight to apply even pressure to the filter paper during the exposure period. The 30-minute sites were exposed in a similar way except that all 30-minute sites on an animal were done at the same time. The 60-minute sites were exposed in a similar way except a rubber tile float was placed on top of the rubber stoppers to ensure even downward pressure. For the 60-minute sites, Vetrap bandaging tape (3M Animal Care Products, St. Paul, MN) was wrapped around the pig to secure the floats in place. At the end of the exposure period, all devices were

removed, and the sites dabbed dry for 30 seconds with a double layer of absorbent Masslin sports towel. The pigs were then placed into a holding cage under an adjacent hood. Once in the holding cage, water was provided ad libitum. Food was provided in the hood within a few hours of recovery from anesthesia. Buprenorphine (0.01 mg/kg intramuscularly) was administered immediately after exposure and early the following morning to alleviate any discomfort. Each pig was kept under the hood for approximately 24 hours. The animals were not placed back into their pens until they tested negative for HD off-gassing using a MINICAMS (O.I. Analytical, CMS Field Products Group, Birmingham, AL).

Postexposure Procedures

At each observation time postexposure (24, 48, and 72 hours), several procedures were conducted. If necessary, lesions were washed with water and dabbed dry. Reflectance colorimetry was conducted followed by LDPI and ICGFI. After the 72-hour measurements, animals were euthanized and skin samples taken for histopathology examination.

Laser Doppler Perfusion Imaging

A moorLDI™ (Moor Instruments, Inc., Wilmington, DE) was used for collecting images. The following operating parameters on the instrument were used: DC gain = 0, flux gain = 0, conc gain = 2, background threshold = 200, distance to subject = 20 cm, scan size = small, scan speed = 10 ms/pixel, DC image resolution = 256×256 , and blood flux units (arbitrary units) set to "perfusion." The scanner head was positioned perpendicular to the wound surface whenever possible. Blood flux levels at each of the pixel points within a defined region of interest (ROI) were measured and averaged, using built-in image analysis software. Each site was analyzed at each time point as follows. Five ROIs were measured within the lesion, each generating an average blood flux value. Those values were averaged, arriving at a single flux value (B) within the burn. Similarly, three ROIs were measured in normal perilesional skin nearest the injury (if available; on a contralateral site otherwise) and averaged, yielding a single flux value (N) for normal skin. A B/N ratio was then calculated. Obvious areas of hyperemia were avoided during the analyses.

ICG Fluorescence Imaging

ICG fluorescence imaging was conducted using a lightweight, portable prototype instrument developed at the U.S. Army Medical Research Institute of Chemical Defense (Aberdeen Proving Ground, MD). Figure 1 shows the wearable diagnostic unit,

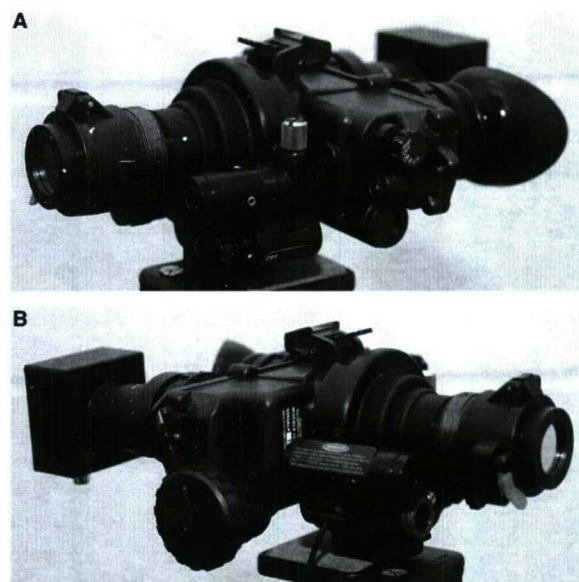


Figure 1. A lightweight, portable prototype ICG fluorescence imaging instrument developed at the U.S. Army Medical Research Institute of Chemical Defense (Aberdeen Proving Ground, MD). It is a wearable diagnostic unit, with views from the left (A) and right (B). The unit consists of night vision goggles equipped with 1) a laser diode for excitation of indocyanine green dye, 2) a filter for highlighting emitted light, and 3) dual aiming beams for setting a consistent distance between unit and patient to keep the fluence of excitation beam constant. A removable charged coupled device camera was inserted in the right optic channel for electronic capture of images.

with views from the left (A) and right (B). The unit consists of night vision goggles equipped with 1) a laser diode for excitation of indocyanine green dye, 2) a filter for highlighting emitted light, and 3) dual aiming beams for setting a consistent distance between unit and patient to keep the fluence of excitation beam constant. A removable charged coupled device (CCD) camera was inserted in the right optic channel for electronic capture of images. In experiments, the dye was excited with the emitting diode, and the resultant fluorescence emission was recorded with the CCD camera. Distance-to-subject was set at 24 inches, thereby standardizing the fluorescence (mJ/cm^2) of the incident light. The optimal distance will need to be determined in future experiments. The images were captured and stored by a computer for analysis at a later time. Image-Pro Plus 4.5 (Media Cybernetics, Silver Spring, MD) was used to perform image analysis. The brightness level of ICG fluorescence from the burns was determined from the images and correlated with the depth of burn injury as determined by histology. These values also were compared with LDPI blood flux determinations. B/N

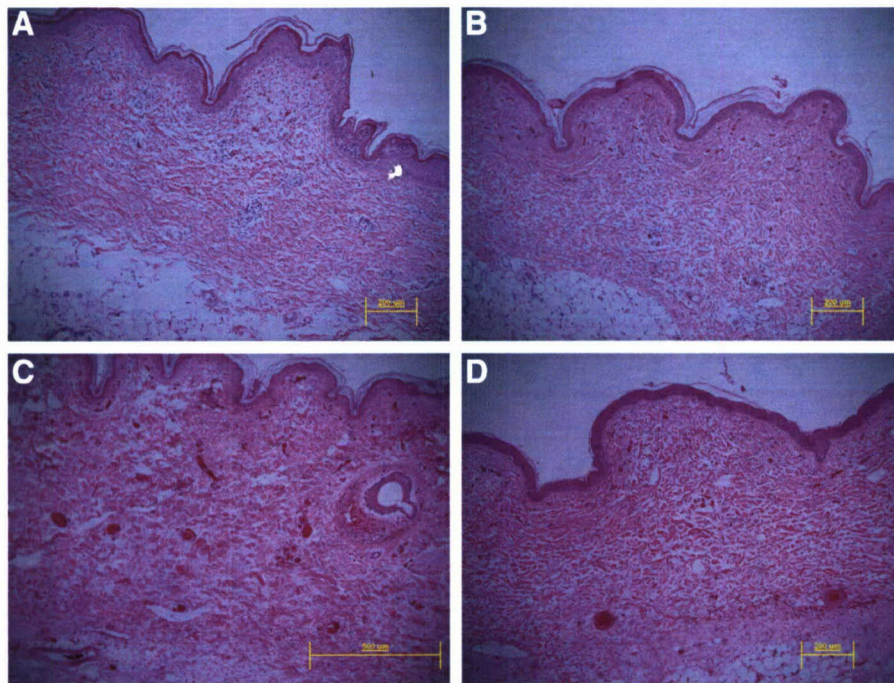


Figure 2. A. Section of skin from the ventral area of weanling swine exposed to 400 μ l of neat sulfur mustard for 2 minutes (hematoxylin and eosin; H&E). Observed minimal focal necrosis of epidermis, minimal multifocal areas of hemorrhage and necrosis in superficial dermis. B. Section of skin from the ventral area of weanling swine exposed to 400 μ l of neat sulfur mustard for 8 minutes (H&E). Observed severe diffuse necrosis of epidermis, severe diffuse necrosis of superficial dermis extending to the fat layer, with mild hemorrhage, and no edema. C. Section of skin from the ventral area of weanling swine exposed to 400 μ l of neat sulfur mustard for 30 minutes (H&E). Observed severe diffuse necrosis of epidermis and dermis with severe hemorrhage and edema extending throughout the deep dermis. D. Section of skin from the ventral area of weanling swine exposed to 400 μ l of neat sulfur mustard for 60 minutes (H&E). Observed severe diffuse necrosis of epidermis and severe diffuse necrosis, hemorrhage and edema throughout the dermis extending to the deep muscle layer.

ratios were calculated in a manner similar to that described previously for LDPI, whenever nearby normal skin was available.

The dose of ICG (Becton Dickinson Microbiology Division, Cockeysville, MD) started at 0.2 mg/kg intravenously but switched to 0.8 mg/kg intravenously after the first animal. It was administered as a bolus through a central or peripheral venous line. The first two range finding pigs were given multiple boli. The total dose of dye injected was kept at less than 2 mg/kg, as recommended by the dye's manufacturer. Uniformity of fluorescence detection was checked each day by recording the fluorescence of a 125-mm diameter disk (Whatman No. 2 filter paper) saturated with a 0.025 mg/ml ICG aqueous solution.

Each observation period included pre-ICG injection photographs of all sites, ICG injection, a 5-minute video on a selected site using high laser, and post-ICG photographs of all sites for up to 30 minutes after ICG injection. A three-photograph sequence was recorded for each site at each time point and included pictures for laser off, laser low, and laser high.

Histopathology

Animals were humanely euthanized after the 72-hour readings. Full-thickness excisions (including panniculus carnosus) of each entire lesion were performed, and surrounding skin was removed, stapled onto labeled, paraffin-coated index cards, and placed into 10% neutral buffered formalin (NBF). To allow adequate NBF into the tissue, two parallel cuts approximately 1.5 cm apart were made in the skin sections before attachment to the index card. Sections were trimmed, paraffin embedded, and stained with hematoxylin and eosin. Evaluation of all sections was conducted in a blinded fashion to determine the depth of the HD lesion.

Statistical Methods

Analyses of the still photo ratios and the LDPI ratios were performed using a two factor repeated measures analysis of variance (ANOVA). The factors were exposure time (2, 8, 30 and 60 minutes) and observation time (24, 48, and 72 hours), which also was the repeated measure factor. If a significant exposure by

observation interaction was observed, then a one factor ANOVA was used at each observation time to compare exposure times. A Tukey's multiple comparison test was used to compare the pairs of exposures and observation times when the main effect was significant. Statistical significance was defined as $P < .05$ for all tests.

RESULTS

A 2-minute HD exposure produced a superficial lesion. Histological evaluation demonstrated minimal focal necrosis of epidermis, minimal multifocal areas of hemorrhage, and necrosis in superficial dermis (Figure 2A). LDPI and ICGFI showed increased blood flow for the 2-minute HD lesion at all observation times (Figure 3). An 8-minute HD exposure produced a lesion of intermediate depth. Histological evaluation demonstrated severe diffuse necrosis of epidermis, severe diffuse necrosis of superficial dermis extending to the fat layer, with mild hemorrhage, and no edema (Figure 2B). LDPI and ICGFI showed

increased blood flow for the 8-minute HD lesion at all observation times (Figure 4). A 30-minute HD exposure produced a deep dermal full-thickness lesion. Histological evaluation demonstrated severe diffuse necrosis of epidermis and dermis with severe hemorrhage and edema extending throughout the deep dermis (Figure 2C). LDPI and ICGFI showed decreased blood flow for the 30-minute HD lesion at all observation times (Figure 5). A 60-minute HD exposure produced a deep full-thickness lesion. Histological evaluation demonstrated severe, diffuse necrosis of epidermis and severe diffuse necrosis, hemorrhage and edema throughout the dermis extending to the deep muscle layer (Figure 2D). LDPI and ICGFI showed decreased blood flow for the 60-minute HD lesion at all observation times (Figure 6).

A summary of the LDPI data is given in Figure 7. This graph gives the mean (\pm SD) blood perfusion ratio comparing lesion skin to normal perilesional skin for HD exposures of 2, 8, 30, and 60 minutes at 24, 48, and 72 hours after exposure.

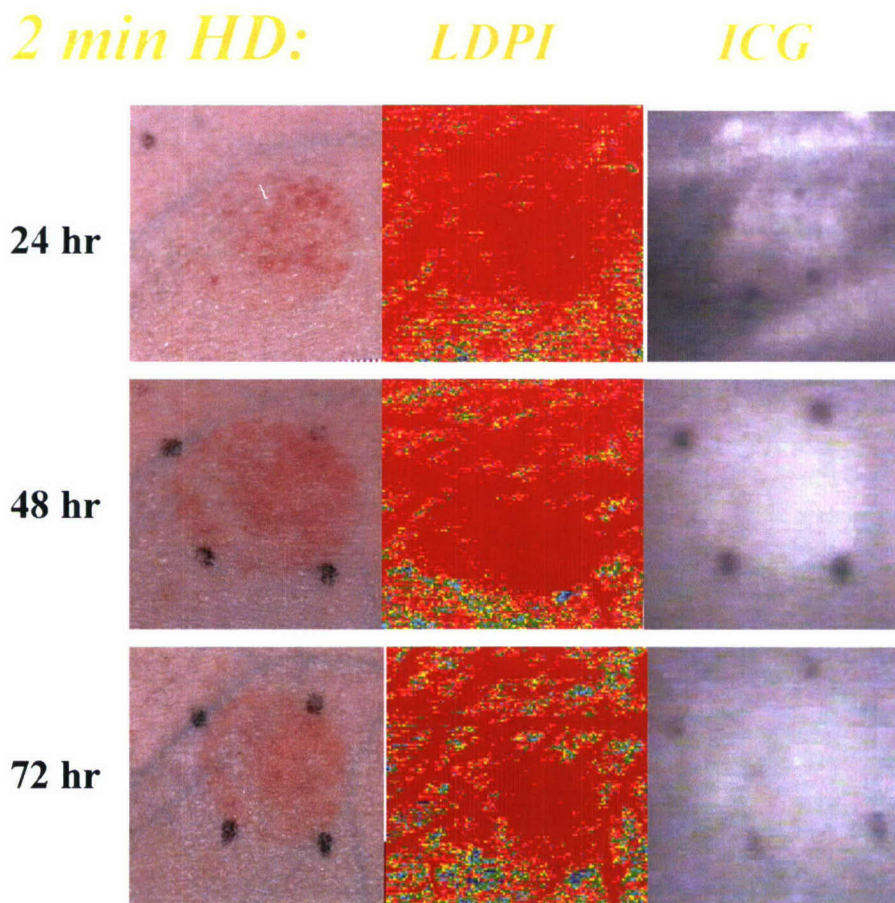


Figure 3. Composite of representative pictures of lesions on weanling swine exposed to 400 μ l of neat sulfur mustard for 2 minutes. Pictures include normal color photograph, laser Doppler perfusion imaging (LDPI), and indocyanine green fluorescence imaging (ICGFI) at 24, 48, and 72 hours after exposure.

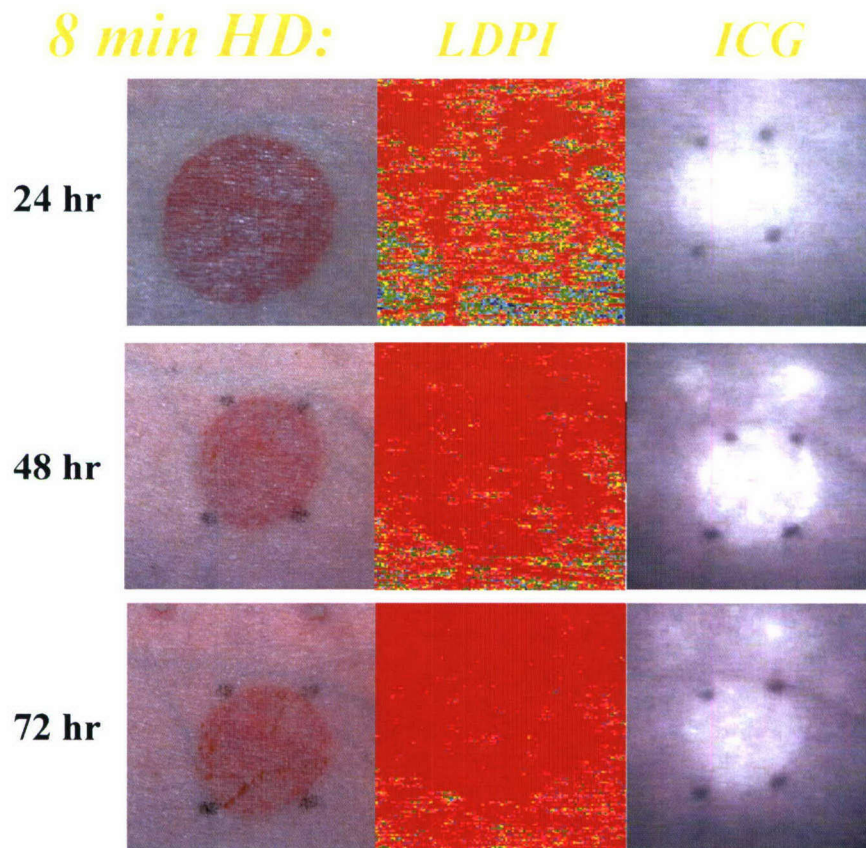


Figure 4. Composite of representative pictures of lesions on weanling swine exposed to 400 μ l of neat sulfur mustard for 8 minutes. Pictures include normal color photograph, laser Doppler perfusion imaging (LDPI), and indocyanine green fluorescence imaging (ICGFI) at 24, 48, and 72 hours after exposure.

A summary of the ICGFI data recorded within 30 seconds of ICG injection is given in Figure 8. This graph gives the mean fluorescence brightness ratio comparing lesion skin with normal perilesional skin for HD exposures of 2, 8, 30, and 60 minutes at 24, 48, and 72 hours after exposure. Not enough sites were available to include meaningful error bars.

A summary of the ICGFI data recorded 10 to 30 minutes after ICG injection is given in Figure 8. This graph gives the mean (\pm SD) fluorescence brightness ratio comparing lesion skin with normal perilesional skin for HD exposures of 2, 8, 30, and 60 minutes at 24, 48, and 72 hours after exposure.

For both the still photo ratios and the LDPI ratios, a significant exposure time by observation time was observed. The significant interaction implies that the relationship of the exposures is dependent on the observation time at which they are observed.

For the still photo ratios, only one borderline significant difference was observed at 24 hours; the 8-minute exposure ratio was less than the 60-minute exposure ($P = .052$).

Analysis of the ICGFI recorded on the video clip shortly after ICG injection (Figure 8) indicated the results reported previously and displayed in Figures 3–6. Image analyses on the still photographs taken 10–30 min after ICG injection are summarized in Figure 9.

For the LDPI ratios, although there was a significant interaction, the relationships of the exposure groups were very similar at the observation times. At 24 hours, 48 and 72 hours, the 30- and 60-minute exposures were significantly different from the 8-minute exposure. At 48 and 72 hours, the 30- and 60-minute exposures were significantly different from the 2-minute exposure, and at 24 hours, the 30-minute exposure was significantly different from the 2-minute exposure.

DISCUSSION

This preliminary study demonstrated that LDPI and ICGFI were useful tools to characterize skin perfusion and provided a good estimate of HD lesion

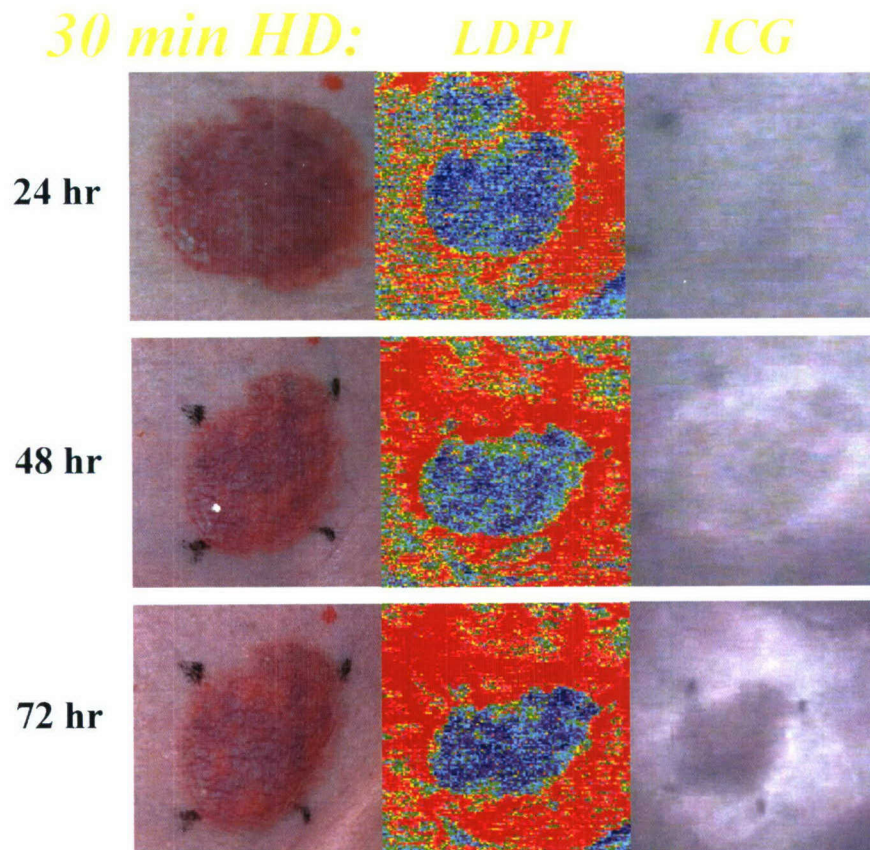


Figure 5. Composite of representative pictures of lesions on weanling swine exposed to 400 μ l of neat sulfur mustard for 30 minutes. Pictures include normal color photograph, laser Doppler perfusion imaging (LDPI), and indocyanine green fluorescence imaging (ICGFI) at 24, 48, and 72 hours after exposure.

depth. Both techniques accurately predicted the need for aggressive treatment (30- and 60-minute HD lesions) and nonaggressive treatment (2- and 8-minute HD lesions) for the lesions generated in this study. The accuracy of these predictions was confirmed by histopathological examination of formalin-fixed, paraffin-embedded skin sections stained with hematoxylin and eosin. Although reflectance colorimetry data also was collected (data not shown) for all sites and observation times, there was no correlation with exposure time or observation time; this technique did not provide any useful information concerning lesion depth.

Further definitive studies will incorporate the use of vital stains such as nitro blue tetrazolium chloride, providing more accurate burn depth determinations via image analysis. On frozen tissues, the reduction of nitro blue tetrazolium chloride by the cell-bound enzyme nicotinamide adenine dinucleotide diaphorase (NADH-diaphorase) leads to an intensive blue granular precipitate (diformazan granules) at the sites of NADH-diaphorase activity. NADH-diaphorase activity has been demonstrated to subside upon cell

death; thus viable (blue) and damaged (unstained) cells can be clearly differentiated.^{29,30}

The LDPI data generated in this study correlate well with previous studies, indicating that the LDPI technique is an excellent tool to help the burn surgeon evaluate the lesion depth. It is difficult, however, to apply this technique in a practical way in a normal hospital setting. The LDPI instrument used in this study is large and difficult to position, though the size of newer models is somewhat less bulky. The important point is that patients must be positioned under the instrument and remain perfectly still for several minutes during the collection period. In addition, each collection is only for a relatively small skin area, depending of the resolution selected.

Relatively few experiments using the ICGFI technique to evaluate burn depth have been reported.^{21,23,24,27} In previous studies, fluorescence was recorded from 5 to 10 minutes after ICG injection. In our studies, using a new prototype instrument, the fluorescence images recorded shortly after ICG injection correlated best for the degree of lesion depth.

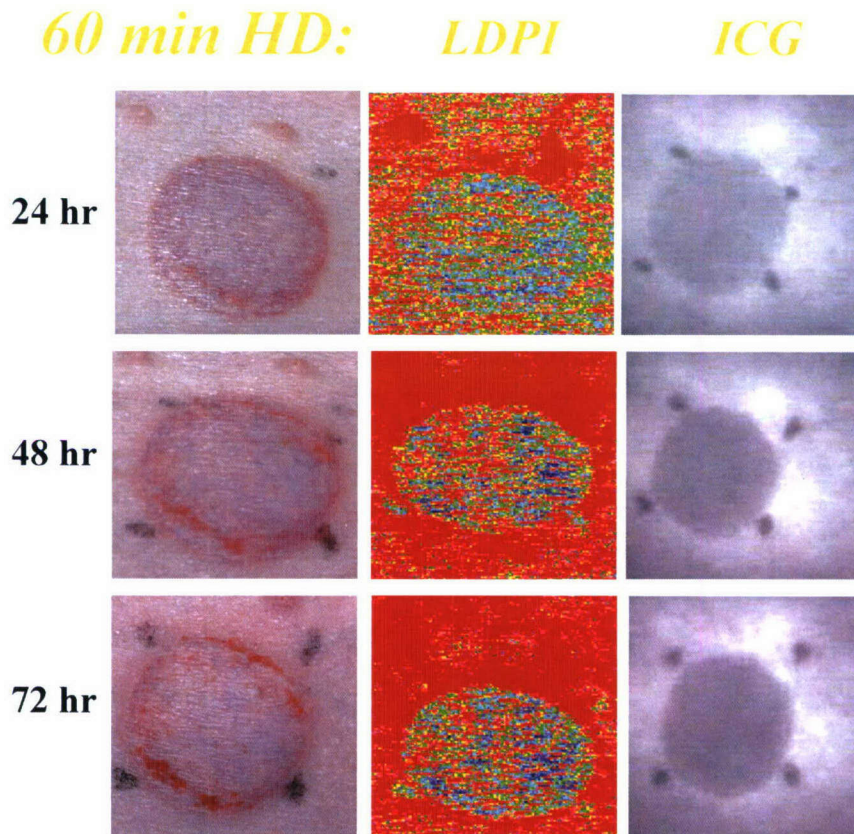


Figure 6. Composite of representative pictures of lesions on weanling swine exposed to 400 μ l of neat sulfur mustard for 60 minutes. Pictures include normal color photograph, laser Doppler perfusion imaging (LDPI), and indocyanine green fluorescence imaging (ICGFI) at 24, 48, and 72 hours after exposure.

The difference in the experimental results may be a function of laser power, ICG injection concentration, or recording distance of the camera. These variables will be evaluated in future studies.

The novel prototype ICGFI instrument used in this study offered several advantages over LDPI and other ICGFI instruments currently available, includ-

ing real-time imaging of blood flow through lesions, cost, small size, portability, and not requiring the patient to be repositioned. A burn surgeon using this prototype instrument could evaluate the entire skin surface in a very short time with only a few injections of ICG. It may be possible, using a CCD video camera, to record the skin fluorescence and reconstruct a

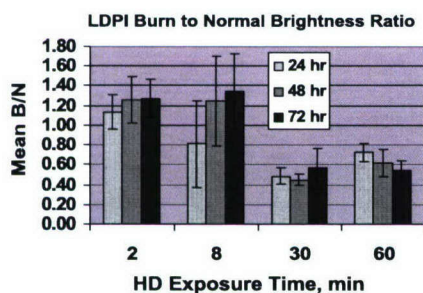


Figure 7. Graph of image analysis giving the mean (\pm SD) laser Doppler perfusion imaging blood perfusion ratio comparing lesion skin with normal perilesional skin for weanling swine exposed to 400 μ l of neat sulfur mustard for 2, 8, 30, and 60 minutes at 24, 48, and 72 hours after exposure.

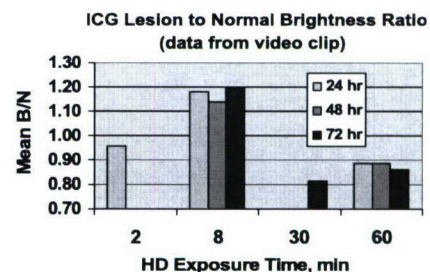


Figure 8. Graph of image analysis giving the mean indocyanine green fluorescence brightness ratio comparing lesion skin with normal perilesional skin for weanling swine exposed to 400 μ l of neat sulfur mustard for 2, 8, 30, and 60 minutes at 24, 48, and 72 hours after exposure. Pictures were taken within 30 seconds of ICG injection.

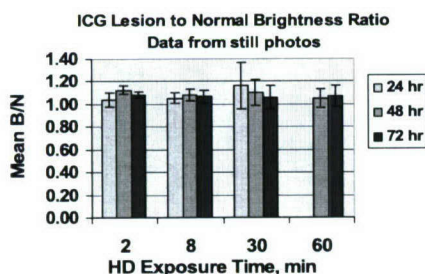


Figure 9. Graph of image analysis giving the mean (\pm SD) indocyanine green fluorescence brightness ratio comparing lesion skin with normal perilesional skin for weanling swine exposed to 400 μ l of neat sulfur mustard for 2, 8, 30, and 60 minutes at 24, 48, and 72 hours after exposure. Pictures were taken 10 to 30 minutes after ICG injection.

digital map of the skin indicating what areas need aggressive treatment and what areas are likely to heal without treatment.

Although proof-of-concept was demonstrated for the prototype ICGFI instrument used in this study, several additional experiments are still needed to completely define the operational use of this technique. Experiments are required to determine the exposure time necessary to produce a graded series of partial-thickness HD lesions. It is important to determine how well this technique will predict the need for aggressive treatment of the truly difficult borderline partial-/full-thickness lesion. Instrument parameters must be optimized for laser strength, standardized viewing distance from patient, and quality of CCD camera. The optimum quantity of ICG used with each injection must be determined along with establishing the best viewing time post injection. Finally, sufficient numbers of animals must be used to establish statistically significant data to accurately define the optimized procedure to be used. The data generated in this and the follow-on study will demonstrate the full potential LDPI and ICGFI hold for predicting the need for aggressive treatment after HD exposure.

CONCLUSIONS

LDPI and ICGFI are useful tools to characterize skin perfusion and provided a good estimate of HD lesion depth. ICGFI has several advantages over LDPI, including rapid real-time viewing of blood perfusion, small instrument size and portability, low cost, and not requiring the patient to be repositioned because the instrument moves instead.

Reflectance colorimetry did not provide an estimate of lesion depth. A 2-minute HD liquid exposure produced a superficial lesion. LDPI and ICGFI showed increased blood flow at all observation times

and provided a good estimate of lesion depth. Aggressive lesion treatment was not indicated.

An 8-minute HD liquid exposure produced a lesion of intermediate depth with severe diffuse necrosis of the superficial dermis. LDPI and ICGFI showed increased blood flow at all observation times and provided a good estimate of lesion depth. Aggressive lesion treatment was not indicated.

A 30-minute HD exposure produced a deep full-thickness lesion with severe diffuse necrosis of epidermis and dermis and severe hemorrhage and edema extending throughout the deep dermis. LDPI and ICGFI showed decreased blood flow at all observation times and provided a good estimate of lesion depth. Aggressive lesion treatment was strongly indicated.

Finally, a 60-minute HD exposure produced a deep full-thickness lesion with severe diffuse necrosis of epidermis and severe diffuse necrosis, hemorrhage and edema throughout the dermis extending to the deep muscle layer. LDPI and ICGFI showed decreased blood flow at all observation times and provided a good estimate of lesion depth. Aggressive lesion treatment was strongly indicated.

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REFERENCES

1. Arturson G. Mechanism of injury. In: Settle JAD, editor. Principles and practice of burns management. New York: Churchill Livingstone; 1996. p. 61–82.
2. Bircher AJ. Laser Doppler measurement of skin blood flux: variation and validation. In: Serup J, Jemec GBE, editors. Handbook of non-invasive methods and the skin. Boca Raton, FL: CRC Press; 1995. p. 399–403.
3. Belcaro G, Nicolaides AN. Laser-Doppler flowmetry: principles of technology and clinical applications. In: Serup J, Jemec GBE, editors. Handbook of non-invasive methods and the skin. Boca Raton: CRC Press; 1995. p. 405–10.
4. Wardell K, Andersson T, Anderson C. Analysis of laser Doppler perfusion images of experimental irritant skin reactions. *Skin Res Technol* 1996;2:149–57.
5. Linden M, Wardell K, Andersson T, Anderson C. High resolution laser Doppler perfusion imaging for the investigation of blood circulatory changes after microdialysis probe insertion. *Skin Res Technol* 1997;3:227–32.
6. Krogstad A-L, Pegenius G, Elam M. Visual scoring and laser Doppler perfusion imaging of skin irritancy induced by different nicotine patches. *Skin Res Technol* 1996;2:158–63.
7. Wardell K, Nilsson G. Laser Doppler imaging of skin. In: Serup J, Jemec GBE, editors. Handbook of non-invasive methods and the skin. Boca Raton: CRC Press; 1995. p. 421–8.
8. Alsbjorn B, Micheels J, Sorensen B. Laser Doppler flowmetry measurements of superficial dermal, deep dermal and subdermal burns. *Scand J Plast Reconstr Surg* 1984;18:75–9.

9. Blomgren I, Bagge U. Postburn blood flow, edema, and survival of the hairy mouse ear after injury at different temperatures. *Scand J Plast Reconstr Surg* 1984;18:269-75.
10. Garner WL, Thomson PD, Moore NP, et al. Effect of triglycyl-lysine-vasopressin on skin blood flow and blood loss during wound excision in patients with burns. *J Burn Care Rehabil* 1993;14:458-60.
11. Micheels J, Alsbjorn B, Sorensen B. Clinical use of laser Doppler flowmetry in a burns unit. *Scand J Plast Reconstr Surg* 1984;18:65-73.
12. Niazi ZBM, Essex TJH, Papini R, Scott D, McLean NR, Black MJM. New laser Doppler scanner, a valuable adjunct in burn depth assessment. *Burns* 1993;19:485-9.
13. O'Reilly TJ, Spence RJ, Taylor RM, Scheulen JJ. Laser Doppler flowmetry evaluation of burn wound depth. *J Burn Care Rehabil* 1989;1-6.
14. Regas FC, Ehrlich HP. Elucidating the vascular response to burns with a new rat model. *J Trauma* 1992;32:557-63.
15. Shakespeare PG. Looking at burn wounds: the A.B. Wallace memorial lecture 1991. *Burns* 1992;18:287-95.
16. Waxman K. Heated laser Doppler flow measurements to determine depth of burn injury. *Am Surg* 1989;June:541-3.
17. Heimbach D, Engrav L, Grube B, Marvin J. Burn depth: a review. *World J Surg* 1992;16:10-5.
18. Brown RFR, Rice P, Bennett NJ. The use of laser Doppler imaging as an aid in clinical management decision making in the treatment of vesicant burns. *Burns* 1998;24:692-8.
19. Chilcott RP, Brown RFR, Rice P. Non-invasive quantification of skin injury resulting from exposure to sulphur mustard and Lewisite vapours. *Burns* 2000;26:245-50.
20. Graham JS, Schomacker KT, Glatter RD, Briscoe CM, Braue EH, Squibb KS. Bioengineering methods employed in the study of wound healing of sulfur mustard burns. *Skin Res Technol* 2002;8:57-69.
21. Green HA, Bua D, Anderson RR, Nishioka NS. Burn depth estimation using indocyanine green fluorescence. *Arch Dermatol* 1992;128:43-9.
22. Black KS, Hewitt CW, Miller DM, et al. Burn depth evaluation with fluorometry: is it really definitive? *J Burn Care Rehabil* 1986;7:313-7.
23. Schomacker KT, Torri A, Sandison DR, Sheridan RL, Nishioka NS. Biodistribution of indocyanine green in a porcine burn model: light and fluorescence microscopy. *J Trauma* 1997;43:813-9.
24. Jerath MR, Schomacker KT, Sheridan RL, Nishioka NS. Burn wound assessment in porcine skin using indocyanine green fluorescence. *J Trauma* 1999;46:1085-8.
25. Holm C, Mayr M, Tegeler J, Becker A, Pfeiffer U, Muhlbauer W. Laser-induced fluorescence of indocyanine green: plastic surgical applications. *Eur J Plast Surg* 2003;26:19-25.
26. Holm C, Mayr M, Hoffer E, Becker A, Pfeiffer UJ, Muhlbauer W. Intraoperative evaluation of skin-flap viability using laser-induced fluorescence of indocyanine green. *Br J Plast Surg* 2002;55:635-44.
27. Sheridan RL, Schomacker KT, Lucchina LC, et al. Burn depth estimation by use of indocyanine green fluorescence: initial human trial. *J Burn Care Rehabil* 1995;16:602-4.
28. Kamolz L-P, Andel H, Haslik W, et al. Indocyanine green video angiographies help to identify burns requiring operation. *Burns* 2003;29:785-91.
29. Neumann RA, Knobler RM, Leonhartsberger H, Gebhart W. Comparative histochemistry of port-wine stains after copper vapor laser (578 nm) and argon laser treatment. *J Invest Dermatol* 1992;99:160-7.
30. Neumann RA, Knobler RM, Leonhartsberger H, Böhler-Sommeregger K, Gebhart W. Histochemical evaluation of the coagulation depth after argon laser impact on a port-wine stain. *Lasers Surg Med* 1991;11:606-15.